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SCE (Sister Chromatid Exchanges) Analysis in Lymphocytes

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16. ABSTRACT

Expectations are high that SCE analyses in lymphocytes removed from individuals in various occupational settings may serve as monitors of preventable hazardous chemical exposures in much the same way that chromosome aberration analyses have been useful for determining irradiation exposures. (SCE is only weakly responsive to ionizing radiations) The enormous complexity of monitoring chemical exposures has not been underestimated: problems shch as diverse confounding exposures, chemical interactions, specific modes of action, and inherent weaknesses in lymphocyte cytogenetic tests(mentioned below) have been addressed. Nonetheless, SCE analyses, along with chromosome aberration tests, may well represent the most feasible and sensitive approaches to this end.

The basic differential staining procedure for assessing SCE in lymphocytes has changed little since its original development. However, the scope of application has been broadened, and particular strengths and weaknesses are now more clearly appreciated. Details concerning the nature of SCE and the general utility of its analysis have been extensively reviewed elsewhere. The intent of this chapter is to focus on methodological considerations which appear to be important to the general understanding and effective application of SCE studies, specifically in lymphocytes.

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SCE ANALYSIS IN LYMPHOCYTES

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SCE ANALYSIS IN LYMPHOCYTES

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TABLE OF CONTENTS

1. INTRODUCTION 3

2. METHODOLOGY 7

2.1. Background 7

2.1.1. Differential Staining for SCE Analysis 7

2.1.2. Lymphocytes: *In Vivo* and *In Vitro* 8

2.2. General Design Considerations 11

2.2.1. Variable Baseline SCE Levels 11

2.2.2. BrdU Dose 12

2.2.3. Combined End Points 13

2.3. Technical 15

2.3.1. Human Blood Cultures and Chromosome Preparations . . 15

2.3.2. Experimental Animal Blood Cultures 18

3. SELECTED APPLICATIONS 19

APPENDIX I 23

APPENDIX II 25

TABLE I 27

REFERENCES 28

INTRODUCTION

Sister chromatid exchanges (SCEs) are generally considered to arise from breakage and recombination of sister chromatid segments at homologous loci.[1] Although the fundamental nature of SCE is not well understood, interests in its frequency have been central both to historical and current studies. Early autoradiographic techniques used to detect this phenomenon were applied for a variety of investigations into its spontaneous and irradiation-related incidences in somatic and germ cells.[2,3] Nearly a decade ago technically simpler bromodeoxyuridine (BrdU)-differential staining methodology was developed in a cultured human lymphocyte system and shown to provide much superior resolving power.[4] Chemical mutagens were clearly demonstrated to induce SCEs at significantly lower doses than those required to cause chromosome aberrations.[5,6] This observation coincided with timely autoradiographic determinations of mutagen action in SCE formation[7,8] and set a new course of emphasis--SCE induction stemming from exposure to environmental agents. BrdU methodology has since been extended to a wide variety of *in vitro* and *in vivo* cellular systems, and hundreds of SCE induction trials have implicated numerous chemical, physical (i.e., UV irradiation), and biological (i.e., virus) agents in the production of this effect.[9,10] A recent summary evaluation of accumulated results has concluded that most chemical carcinogens induce SCEs, the test being particularly sensitive to agents which cause DNA adducts.[1]

The molecular mechanism of SCE formation is uncertain. Although SCEs bear some cytological likeness to meiotic crossover products and to certain forms of chromosome aberration and are biochemically close to various steps

or consequences of DNA repair, there is no strong evidence in support of close, direct relationships to these processes.[9-12] On the contrary, SCE is generally viewed as a unique DNA event which, when induced, arises from some small fraction of the actual number of lesions.[10,13] Its mechanics of formation apparently occur during DNA synthesis regardless of when the lesion was induced.[7] Evidence and theories favoring a critical involvement of the replication fork are mounting. (For review, see Stetka[12]). Painter[14] has proposed an attractive model, which depicts SCEs as arising from unrepaired damage at junctions between replicon clusters. These sites are suggested to be especially susceptible to double strand breakage, with subsequent recombination between parent and daughter strands leading to SCE. An alternative model,[15] with supporting evidence, depicts SCEs as arising from breakage following progression of the replication fork: Errors on the part of enzymes involved in replicated DNA unravelling processes then lead to sister chromatid recombinations.

The biological significance of SCE to the cell is also uncertain. SCEs are always detectable in control samples (although these may arise from the techniques used in the study), and increases in their frequency due to mutagen exposure is not known to directly affect cell viability or function. A linear relationship between induced SCEs and gene mutations (HPRT locus) in mammalian cells has been shown.[16-18] However, the relative efficiencies of these genetic end points (and of chromosome aberrations as well) is chemical-specific and may vary widely with different modes of chemical-DNA interaction.[16-19] Nevertheless, SCE remains an extraordinarily sensitive measure of DNA alteration by most mutagenic chemicals and is recognized as usually paralleling clastogenic and gene

mutation events. Consequently, SCE both in its own right, and in signalling the potential for more classical forms of genetic damage to which it is often a corollary, has become a major indicator of the genotoxic potential of chemicals.

Within experimental and human monitoring contexts, lymphocytes provide a convenient and relevant tissue for SCE analyses. They are easy to obtain and grow in culture, and in the laboratory they have proven metabolically competent to activate certain mutagens to induce SCEs.[20,22] Expectations are high that SCE analyses in lymphocytes removed from individuals in various occupational settings may serve as monitors of preventable hazardous chemical exposures in much the same way that chromosome aberration analyses have been useful for determining irradiation exposures.[9,23-25] (SCE is only weakly responsive to ionizing radiations.[6]) The enormous complexity of monitoring chemical exposures has not been underestimated: Problems such as diverse confounding exposures, chemical interactions, specific modes of action, and inherent weaknesses in lymphocyte cytogenetic tests (mentioned below) have been addressed. Nonetheless, SCE analyses, along with chromosome aberration tests, may well represent the most feasible and sensitive approaches to this end.

The basic differential staining procedure for assessing SCE in lymphocytes has changed little since its original development.[4] However, the scope of application has been broadened, and particular strengths and weaknesses are now more clearly appreciated. Details concerning the nature of SCE and the general utility of its analysis have been extensively

reviewed elsewhere.[1,9,10] The intent of this chapter is to focus on methodological considerations which appear to be important to the general understanding and effective application of SCE studies, specifically in lymphocytes. A short discussion of some selected results is also provided.

2. METHODOLOGY

2.1 Background

2.1.1. Differential Staining for SCE Analysis

SCEs do not appear to involve inequalities in amounts of exchanged material, and there is no gross morphological evidence of their occurrence. Thus, some means of differentiating the sister chromatids is typically required for detecting these exchanges. Using [^{3}H]-thymidine as a DNA label, and following its semiconservative pattern of distribution through successive replication periods, Taylor et al.,(27) first delineated SCEs in second division metaphase cells. The exchanges appeared as sites of coincident alternation in grain patterns between the sister chromatids. Higher resolution differential staining methodology, which has now largely supplanted autoradiography for SCE analysis, relies upon the substitution of BrdU for thymidine in the DNA. Labelling over both, or alternatively only the first, of two consecutive DNA synthesis periods results in an equal distribution of BrdU to the sister chromatids of first subsequent metaphase stages and in an unequal distribution at second metaphase stages (Fig. 1). This asymmetrical presence of analogue in second metaphase sister chromatids causes their differential appearance when chromosomes are stained with a BrdU-sensitive dye. For example, BrdU quenches the fluorescence of 33258 Hoechst dye such that the more heavily substituted chromatid appears dull in contrast to the brighter chromatid with relatively less analogue substitution.(4) SCEs are then clearly detectable as reciprocal staining discontinuities. However, since most fluorescence methods suffer from rapid

fading, SCE analysis is more often performed on cells which are additionally processed through photolysis and Giemsa overlay steps[28,29] to become permanent differential staining preparations (Fig. 2). Mean SCE frequencies for treatment groups of cells are statistically compared with those of control cell samples. Details concerning preparative and analytical methods for SCE analysis are comprehensively provided in a number of reviews,[1,9,10,23] and examples are given below. Although cell tolerance of BrdU uptake and escape from toxic effects may be somewhat variable between somatic, germ, and certain embryo tissues, this is not generally a problem in lymphocyte protocols. Rather, the basic concern in the latter tissue is simply the occurrence of sufficient cell division for the sequential steps of DNA labelling, cell cycling, and chromosome staining to be carried out.

2.1.2. Lymphocytes: *In Vivo* and *In Vitro*

Comprehensive reviews of lymphocytes and their activation characteristics are provided by Ling and Kay[30] and Greaves et al.[31] Aside from peripheral blood, lymphocytes are enmassed in a number of organs: spleen, thymus, bone marrow, lymph nodes, gastrointestinal tissue, tonsils, and appendix. In rodent experimental systems, several of these tissues have been analyzed for SCE[32]; the very high mitotic index of hematopoietic cell types in bone marrow has made it an especially routine tissue choice. The accessibility of peripheral blood primarily accounts for its selection as the usual source of lymphocytes for human cell experimentation and monitoring (and is similarly advantageous for experimental animal studies). In human blood, multigram quantities of lymphocytes are circulating

throughout the body[30] and to various organs where specific genotoxic metabolites may be generated. These are, for the most part, marrow-derived, differentiated cell types in the nondividing G_0-cell-cycle stage except when involved in immune reactions. Their life span in blood is variable--up to many years. If plant lectins or other agglutinating mitogens of cultured lymphocytes are delivered *in vivo*, there is not a reliable increase in the yield of recoverable dividing cells. On the contrary, leukopenia may result from splenic corralling of the affected cells.[30]

SCE analyses in lymphocytes rely upon *in vitro* cell cycling and BrdU incorporation. Several such studies have utilized lymphoblastoid cells,[33-36] usually of permanent cell lines derived from patients with lymphoproliferative disorders. Systems employing exogeneous metabolic activation have included the use of rat liver S9 mix[35] and the implantation of diffusion chambers of lymphoblastoid cells into mice.[33] Lymphocytes from normal individuals remain mitotically quiescent in culture unless a stimulatory agent is added. Phytohemagglutinin (PHA), an extract of the red kidney bean *Phaseolus vulgaris*, is most often used for this purpose. The initiating mechanism is believed to involve mitogen binding to cell surface receptors--a process bearing some resemblance to antigen-induced immune reactions.[30] That metabolic changes are known to accompany this process[30] is further evidenced by transformation--related enhancement of chemical activation to induce SCEs.[38] Primary lymphocyte cultures are short-term; cell division ceasing within 1 to 2 weeks.[30,37]

In mice, the PHA response appears to be under multifactorial genetic control. C57 and DBA strains are among the highest and lowest responders,

respectively.[39] In certain human disease conditions, impaired levels or deviant patterns of lymphocyte responsiveness to PHA are encountered.[40,41] Normally, however, the number of cells responding depends upon the concentration of PHA.[30,37] The earliest replicators begin their DNA synthesis approximately 24 hr after stimulation; others asynchronously up to 4 days or longer after PHA treatment.[30] Thus, while metaphase cells harvested at approximately 42 hr represent mainly first division cells,[37,42] those at considerably later times may represent mixtures of cells in their first, second, third, etc., division. A high proportion of second division cells may be analyzed at 72 hr.[1,37,43] Cell-cycle times of 12 to 16 hr have been estimated or measured.[37,45] T-lymphocytes are considered the primary responders to PHA[30,31]; although smaller fractions of B-lymphocytes also proliferate.[44,45] Reportedly, the latter cells respond to PHA more slowly[45], cycle faster,[43] and appear with increasing proportional representation over time.[45] At least one study has determined B-cells to have a lower baseline SCE frequency than T-cells.[43] However, conflicting information exists concerning differences in SCE frequency for fast- and slow-growing or PHA-responding cells.[43,45-50] The actual extent to which variations in lymphocyte cell-type and/or proliferation characteristics may be affecting SCE frequencies is not well understood.

2.2 General Design Considerations (Technical Problems, Scope)

2.2.1. Variable Baseline SCE Levels

Even when technical steps such as culturing conditions and time of cell harvest are held tightly constant, lymphocytes from different individuals, as well as from the same individual over time, may exhibit considerable variability in baseline and inducible SCE levels.(47) Some potential sources of variation are conjectural and may involve subtle differences in metabolism, DNA repair, or lymphocyte history of exposure. Others may pertain to individual characteristics which are routinely describable but as yet insufficiently studied to draw firm conclusions as to their importance. For example, evidence to date indicates that age and sex are not significant influences upon SCE frequencies in normal human lymphocytes.(25,49) However, experimental studies have shown that cells from older human and animal subjects tend to exhibit a relatively lower SCE response to chemical clastogens than their younger counterparts.(51,52) It has also been reported that male and female benzene-exposed mice experience markedly different levels of SCE induction in bone marrow cells.(53) Several studies are in agreement that smoking can elevate lymphocyte SCE levels.(21,25,54,67) In view of the early stages of SCE monitoring in humans, it seems generally advisable to record all potentially relevant information which is easily obtainable through personal histories, as well as to ascertain basic hematolgoical data from routine blood differential analyses. Time-dependent increases in SCE (from storage)(45) or decreases due to repair or dilution of DNA effects leading to SCE (see Selected Applications section below) may occur. Thus, the time interval between exposure to a potential SCE-inducing

substance and harvest of cells is critical to experimental design and interpretations.

Typical of SCE assays in general is the relatively wide range of individual cell deviations from the mean SCE frequency of a sample. (See Table I.) Whether this is due to cell differences in BrdU uptake or past mutagen exposures is uncertain. In any case, experimentally, it is not readily controllable. Wide SCE ranges in control and exposure cell populations are manageable through adjustment of the cohort and/or cell sample size for analysis. Generally, scoring increasing numbers of individuals and cells (i.e., upwards from 25/culture) enables detectability of smaller SCE increments. An account of outlier cells (with very high numbers of SCEs) through assessment of individual distributions of SCEs is also advised.(23)

2.2.2. BrdU Dose

It is well established that BrdU induces exchanges.(1,9) However, it is not entirely clear to what extent this may relate to analogue substitution in DNA or to a primary effect at a non-DNA site, i.e., interference with deoxycytidine metabolism.(55) Regardless, the amount of BrdU available to the cells can influence baseline SCE levels. Thus, it has been widely recommended that individual laboratories engaged in lymphocyte SCE monitoring standardize the BrdU concentration to cell number ratio per culture.(1,23,47) An appropriate ratio may be determined from the establishment of a BrdU-dose/SCE-response curve for constant cell number and

culturing conditions. Special attention should be given to maintaining consistency of serum type (and perferably batch) and to eliminating all exposure to light of certain wavelengths, since these are variables which have been clearly shown to influence SCE formation.[1,23] An optimal BrdU dose is generally considered to be one which 1) provides high quality differential-staining of sister chromatids, 2) does not markedly inhibit cell replication or cycling rates, and 3) results in an appropriate baseline SCE frequency. The latter may be interpreted in different ways. One line of reasoning holds that an SCE frequency falling in the plateau region of the curve (at higher BrdU concentrations) is most suitable, in that the impact of SCE fluctuations stemming from other procedure-related influences will be minimized.[47] Others may contend that the lowest SCE frequency consistent with reliable, high quality staining results is best, because it provides for maximum assay sensitivity in detecting small SCE increases. Further insight into the potential importance of these and other technical factors is provided in a thorough study by Carrano et al.[47]

2.3.3. Combined End Points

SCE's in human lymphocytes are often measured within parallel *in vivo*-*in vitro* approaches, or in conjunction with other genetic or toxic end points. *In vitro* testing has helped to confirm that negative cytogenetic findings after *in vivo* exposures were due to chemical unreactivity rather than pharmacokinetic factors or repair characteristics of the organism.[56,57] Similarly, chemical exposure trials in culture may help clarify metabolic requirements and activities when positive *in vivo* results

are obtained. Correlated incidences of mitomycin-C-induced SCEs and gene mutations (HPRT) were recently demonstrated in parallel lymphocyte cultures evaluated for SCE and 8-azaguanine resistance.[18] Cell proliferation and chromosome aberrations may be assessed from the same cultures--and even the same slides--providing SCE information. Since chromosome stain patterns reflect the number of divisions undergone subsequent to BrdU exposure, ratios of first, second and third (or higher) division cells occuring may be determined along with SCE frequencies. Cytotoxicity in the form of increasing proportions of first division cells (corresponding to decreasing incidences of later-division cells) has been noted to accompany SCE induction.[49,53,68] The capability to unequivocally identify and analyze only first division metaphase cells for chromosome aberrations greatly enhances the sensitivity of that assay, since damage which is in cells with inhibited cycling, or subject to dilution or altered expression by cycling, will not be missed.[26] A number of laboratories are presently conducting large-scale studies to determine any disadvantages or potential inaccuracies attached to the analysis of chromosome aberrations in cells substituted with BrdU.

2.3 Technical

2.3.1. Human Blood Cultures and Chromosome Preparations

Lymphocyte procedures for SCE analysis follow several common basic steps (Fig. 3), although with considerable flexibility in technical detail. This section is summarized from the authors' experience and from several current published works[1,9,23,26,47] which specifically address this topic. In human monitoring studies, blood is obtained by venipuncture, mixed with an anticoagulant such as heparin or acid-citrate-dextrose solution, and set up as sterile replicate cultures of either whole blood or leukocytes obtained after sedimentation or purification on a density gradient such as Ficoll-Hypaque. In our experience, it has proven advantageous to culture blood which was fresh (hours old) as opposed to stored, the latter having exhibited reduced leukocyte recovery. Culture media commonly used include RPMI 1640, Eagle's MEM, or HAM's F-10, with 15 to 20% fetal bovine serum and antibiotics. Reagent grade PHA is generally recommended over the purified form, in that it has a wider dose range of effectiveness, less cytotoxicity, and is more economical.[30] Poor PHA responsiveness often characterizing the lymphocytes of cancer patients has been noted to very significantly improve if cells are washed successively in media prior to culturing with PHA.[58]

Typically, 5 or 10 ml cultures are set up in >15 ml tubes or flasks (glass or plastic). Cultures should be standardized for the number of cells and for the BrdU concentration. White cells, usually counted with a Coulter counter, cytograf, or hemacytometer, are commonly grown in concentrations

of 5 to 8 x 10^6 cells/culture. BrdU, generally used within the range of 10 to 100 μM concentrations (25 μM is often selected), may be added at the time of culture initiation or after 24 h, when the first DNA synthesis staging is beginning. Suspension cultures are positioned upright or on a slant in a 37°C, 5% CO_2 incubator. The closely settled cells should be disturbed as little as possible: Continuous agitation inhibits PHA activation.(30) Because light wavelengths <313 nm can degrade BrdU-substituted DNA and induce SCEs,(10) cell cultures should be completely protected from these effects, e.g., by using light-tight incubators and yellow extraneous lighting conditions. Although lymphocyte cycling rates may vary on an individual basis, 72 h cultures have been found to routinely provide high proportions of second division metaphase cells. A spindle inhibitor such as Colchicine or Colcemid is added at 68 to 70 h in order to halt and collect metaphase stage cells. Standard cytogenetic harvest procedures which are based upon hypotonic and successive fixative treatments are employed. The most commonly used slide-staining and analysis procedures are those utilizing 33258 Hoechst fluorescence or fluorescence plus Giemsa techniques.(1) A detailed procedure for growing human lymphocytes and for differentially staining chromosomes which has yielded superior results in our laboratory, is provided in Appendix I.

Lymphocytes are grown in a similar manner for the purpose of _in vitro_ SCE induction tests. A variety of dosing and metabolic activation procedures are effective--the optimal choice depending upon the totality of experimental design and intent considerations. The Gene-Tox Program Report on SCEs(1) has made a number of recommendations concerning _in vitro_ chemical testing in human lymphocytes, many of which are enumerated below. It is

desirable to obtain information on effects at G_0 and S stages. Exposure might be conducted prior to, at the time of, or at various times (i.e., 24 or 48 h) after PHA stimulation. The inclusion of S9 mixture prepared from rat livers[59] has proven effective as a means of adding metabolic activation capabilities; under these conditions, toxicity and optimal serum concentrations, exposure times, and exposure durations have been addressed.[1,59] In the absence of exogenous activation, continuous exposure over the last 24 h of culturing conveniently accomplishes insult during the entirety of the last cell cycle. It is further recommended that at least 3 doses covering 2 logs be administered. The highest dose should be at the limits of solubility or produce toxic effects in the form of reduced mitotic indices, chromosome aberrations, or delayed cycling to result in shifts to relatively higher proportions of first metaphase division cells.

Commonly used solvents or carriers have not generally been implicated in SCE induction, although negative control cultures with and without the solvent should always be performed.[1] Effective positive control chemicals include cyclophosphamide with S9 for chemicals requiring metabolic activation, and ethyl methanesulfonate or mitomycin C as direct-acting agents. Slides should be coded and scored blind (methods of scoring are illustrated in Bloom et al., 1981,[23]) and SCE frequencies reported on a normalized diploid complement basis. The number of cells analyzed and their statistical treatment depends upon sensitivity requirements and experimental design. A common practice has been the application of T-tests for evaluating mean SCE frequency results based upon samples of 25 or more cells/replicate culture (total = 50+ cells/group); the test data are, in

some cases, subjected to log or square root transformation to achieve stabilization of variances. Some guidance concerning appropriate interpretations and conclusions in various instances of equivocal results is provided in the Gene-Tox Report on SCEs.[1]

2.3.2. Experimental Animal Blood Cultures

Basically similar protocols have been followed for SCE analysis in peripheral blood lymphocytes from a variety of experimental animals. Specifically, rabbit,[60] cattle, pig, and sheep,[61] and rat[62,63] systems are among those reported. Rabbit and rat systems have been used for *in vivo* and *in vitro* SCE induction analyses. Kligerman et al.[63] have thoroughly investigated the conditions for optimal growth and analysis of rat lymphocytes. We have found that their basic procedure provides excellent results: A slightly modified version of it which we routinely employ for *in vivo* and *in vitro* SCE induction trials is notable in Appendix II.

3. SELECTED APPLICATIONS

The broad utility of lymphocyte SCE systems is evident in their diverse human monitoring and experimental applications and in their extensive representation within surveys of chemical mutagenesis trials. In addition to their use to signal genetic effects from occupational or medical exposures to various agents, SCE analyses have been used to identify and characterize chromosomal instabilities in cancer-prone individuals. (For review, see Galloway and Tice.[26]) There is also current interest in the prospect that studies of changing SCE frequencies in the blood of cancer chemotherapy patients may prove instructive towards the proper selection of therapeutic agents and treatment regimens.[64] Lymphocyte SCE induction has been used to interpret cell metabolic and DNA repair capabilities[21,22,65-68] and to evaluate the effects of different agents' interacting to result in modified mutagenic responses.[65] The Gene-Tox Report[1] lists more than 45 different chemicals evaluated for their capabilities to induce SCEs--in some cases relative to efficiencies in the production of other forms of genetic damage. A sample of experimental data from the authors' laboratory, which was obtained with the appendixed protocols, is noted below.

The well known animal carcinogen, ethyl carbamate (urethane), causes extensive SCE induction when injected into mice.[69,70] Yet, in cell culture studies it is considered a "problem" chemical[71] because of its generally weak mutagenicity in such systems.[72] As some other substances thought to require metabolic activation to induce SCEs have proven positive for this effect in human lymphocytes without added activation

capabilities,[21,22] we, and others,[73] have examined ethyl carbamate as well as vinyl carbamate for SCE induction under these circumstances. Vinyl carbamate is a suspect metabolite of ethyl carbamate and is considerably more carcinogenic.[74] Although it has recently been reported that ethyl carbamate is more active than vinyl carbamate for SCE induction and can cause a tripling of control-level SCE values in cultured lymphocytes,[73] we have been unable to confirm this (Table I). Over a broad dose range, with inhibited cell replication evident at the highest dose, ethyl carbamate was completely negative for SCE induction. On the other hand, vinyl carbamate caused increases in SCE frequencies that were more than 5 times that of controls. These findings are consistent with our observations of ethyl carbamate negative activity in cultured mouse marrow cells[69] and Chinese hamster V-79 cells,[72] as well as vinyl carbamate positive activity in V-79 cells[72] and also in rat lymphocytes removed for culturing after *in vivo* exposure (unpublished observations). Despite some seemingly unique metabolic capabilities of human lymphocytes,[21,22] we conclude that ethyl carbamate remains negative for SCE induction although vinyl carbamate is clearly efficient for producing this effect. The discrepancy for these chemicals between our findings and those referenced above is not readily explainable, and it exemplifies the inconsistencies alluded to earlier which may sometimes characterize this assay and which may result from any of a number of possible technical reasons. Individual differences in lymphocyte metabolic capabilities may have influenced the conflicting results, although repeat studies in our laboratory using different donors have confirmed our original findings.

The rather small number of human monitoring SCE trials which have thus far been conducted confirm the promise of this approach for detecting genotoxic exposures and also point to important limitations in its application. Some agents which have given positive *in vivo* SCE test results in human blood cells include various cancer chemotherapeutics[1,9,10,26,67] and laboratory chemicals--e.g., solvents,[75] nalidixic acid,[76] vinyl chloride,[77] and ethylene oxide.[25,78] However, in some circumstances, the sensitivity of SCEs was less than that of apparently longer-lived chromosome aberrations in revealing genetic effects.[56] In general, it has been found that acute *in vivo* exposures to SCE-active agents give rise to transient induction levels detectable for only days or weeks (an exception is noted below). With repeated dosing, initially high SCE increments tend to fall to more stable intermediate levels of increase before returning to normal frequencies some weeks to months later. These patterns initially reported in experiments with rabbits[60] have been observed in human studies involving different chemicals[64,79]; and reports of negative results are consistent with this as well.[80] Thus, a reasonable conclusion is that the reliability of SCE analysis may be largely limited to instances of high chronic exposure or to assessments immediately (within days) following an acute exposure.[56] Possible explanations for the rapid decline in SCE levels include the death of damaged cells, their dilution by new undamaged cells, and the repair of damage.[60,64,79]

Although peripheral lymphocytes *in vivo* are considered to be relatively inefficient at excising damaged DNA,[30] some repair ability is suggested by recent observations that methyl methanesulfonate and nitrogen mustard produce significantly more SCEs in cultured human lymphocytes when treatment

is at late G_1,-S than when it is at early G_1.[67] An unusual SCE induction response has been noted in lymphocytes from cancer patients treated with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.[67] A single dose of this agent results in a significant elevation of SCEs, which is detectable for 2 months. It is suggested that slow forming cross-links may be occurring to more than offset repair removal of SCE-related mono-adducts.[67,68] Lymphocyte SCE analysis has clearly led to some interesting findings in regard to potential chemical-DNA interactions. The sensitivity of this assay to reveal genetic effects, combined with its versatility within experimental systems and relevance for human monitoring, should assure its continued application in matters of genetic health hazard assessment.

APPENDIX I: A PROCEDURE FOR GROWING AND PREPARING HUMAN LYMPHOCYTES FOR SCE ANALYSIS.

1. Blood obtained by venipuncture is collected and gently mixed in 10 cm^3 sterile vacutainer tubes containing 143 units of sodium heparin (Becton-Dickinson).

2. A sample of 0.2 ml is removed for determination of the WBC concentration per milliliter (and optional differential cell count analysis). A Coulter counter with 100 µm aperture tube and Zap-O-Globin (Coulter Electronics) to lyse red blood cells is conveniently used.

3. Added to replicate, sterile 25 mm^3 culture flasks (Corning) are 5×10^{-6} WBC (typically in 0.7 to 0.9 ml of blood), along with culture medium, PHA, and BrdU, to give a total of 10 ml. The medium consists of RPMI 1640 with 15% fetal bovine serum and 100 units/ml of Penicillin G + 100 µg/ml streptomycin (GIBCO). Reagent grade PHA (Burroughs-Wellcome, HA-15) at 0.15 ml/culture and 25 µM BrdU (Sigma) are used.

4. Flasks are incubated upright for 72 hr under the conditions of 37°C, 5% CO_2, and complete absence of light. Cells are not disturbed except for the addition of 2.5 µg Colcemid (Sigma) to each flask at 70 hr or for the introduction of a mutagen at one of several optional times (see text) for <u>in vitro</u> testing. These latter steps are carried out under yellow room lighting. (See text.)

5. Cells are transferred into centrifuge tubes, spun at 400 g for 5 min, and the cell pellet resuspended in several milliliters of 0.075 M KCl hypotonic solution for 10 min. Cells are then repeatedly fixed (3 times) in 3 parts methanol:1 part acetic acid (freshly prepared).

6. Drops of cell suspension are applied to cold wet slides and allowed to air-dry. After staining with 50 µg/ml of Hoechst 33258 dye (Behring Diagnostics, Amer. Hoechst Corp.) in distilled H_2O (pH 7) for 10 min, slides are H_2O-rinsed and layered with McIlvanies buffer at pH 7, coverslipped, and subjected to black light (General Electric, F40B1) photolysis at a distance of 2 inches for 20 min. During this time, slides are on a warmer tray at 50°C. Following a rinse in distilled H_2O, slides are immersed in 4% Giemsa dye prepared with 1 part McIlvaines buffer:4 parts H_2O, pH 7; the slides are rinsed in H_2O again and allowed to dry for subsequent light microscope analysis.

APPENDIX II: A PROCEDURE FOR GROWING AND PREPARING RAT LYMPHOCYTES FOR SCE ANALYSIS (adapted from Kligerman et al., 1981[63])

1. Blood is obtained from the heart or tail vein[81] of CD-1 rats (Charles River). Heart punctures are performed with sterile 5-10 ml heparinized (approx. 10 units/ml) syringes (Becton-Dickinson) + 23G needles in etherized animals. Approximately 5 ml of blood is routinely obtained. Alternatively, the animal is warmed under a heat lamp to achieve vasodilatation, and a small razor-cut made in the underside (after alcohol swabbing) approximately 1 inch from the base; or, the distal 0.5 inches of the tail may be snipped. After discarding the first 2-3 drops of blood, 10-12 drops are collected in a small sterile tube containing 50 units of sodium heparin (Sigma). Blood and heparin are gently mixed.

2. Heart blood is transferrred to a 15 ml centrifuge tube where it is spun at 500 g for 5 min. Washing three times with phosphate-buffered saline (GIBCO), pH 7.4, is advised, as this reportedly provides improved results.[63] Cells are resuspended in medium to a total volume of 5 ml. (Tail vein blood [0.2 ml], optionally washed, is combined with 1.8 ml medium.) Culture media consist of RPMI 1640 with 25 mM Hepes buffer and 2 x L-glutamine, 10% heat-inactivated fetal bovine serum (GIBCO), and 50 µg/ml gentamicin sulfate (Schering Corp.). Ten units per milliliter of preservative-free sodium heparin (Sigma) is also added. Purified PHA (Burroughs-Wellcome, HA-16; 8 µg/ml culture) or reagent grade

(0.1 ml/culture) PHA (Burroughs-Wellcome, HA-15) has provided excellent mitotic stimulation results. The PHA reactivity may be checked, since it is known to vary among lots.(81)

3. Samples of 0.6 ml heart blood cell suspension (in media) are transferred to 15 ml sterile culture tubes (Falcon) and combined with additional complete medium to give 5.4 ml culture. (The 2.0 ml tail blood cultures are not diluted.) After incubation for 6 hr at 37°, 5% CO_2, the cell culture is gently mixed, and cells are returned to the incubator for an additional 18 hr. The complete medium is changed and BrdU added to give a final concentration of 10 µM. Cultures are reincubated for an additional 28 to 30 hr in the complete absence of light. Colcemid (Sigma) at 0.05 µg/ml is introduced for the last 3 hr. These lymphocyte culturing and BrdU substitution conditions are suitable for *in vivo* or *in vitro* mutagen exposure experimentation. Cytogenetic techniques for cell harvest, and slide preparation and staining are as described in Steps 5 and 6 of Appendix I.

Table I. In Vitro SCE Frequencies in Human Lymphocytes Treated with Ethyl Carbamate or Vinyl Carbamate

Test Chemical	Dose (mg/ml)	Number of Cells	SCE/Cell Ranges	SCE Frequencies[a]
Control	-[b]	54	3-14	7.4 ± 2.7
Ethyl Carbamate	1.0	65	2-15	8.3 ± 3.0
	5.0	54	4-14	7.9 ± 2.7
	10.0	34	3-20	8.2 ± 4.2
Vinyl Carbamate	0.01	31	3-17	9.1 ± 3.8
	0.1	27	17-67	41.4 ± 12.4
	0.2	28	17-66	39.3 ± 12.5

[a]Mean value ± standard deviation/sample of cells analyzed.

[b]All control and treatment group cells were grown with 25 μM BrdU.

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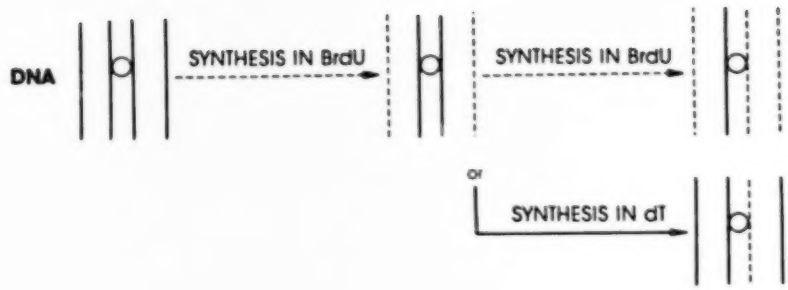

Figure 1. Diagrammatic representation of asymmetrical BrdU substitution in sister chromatids as achieved by alternative labelling procedures over two consecutive DNA synthesis periods. BrdU incorporation (broken lines) during the first replication period gives rise to first division chromosomes which are unifilarly substituted in both chromatids. An additional replication with BrdU leads to unifilarly vs. bifilarly substituted second-division sister chromatids. In the absence of BrdU label during the latter replication, endogeneous deoxythymidine is utilized (solid lines), and only one of the resulting sister chromatids will contain the analogue (unifilarly substituted.)

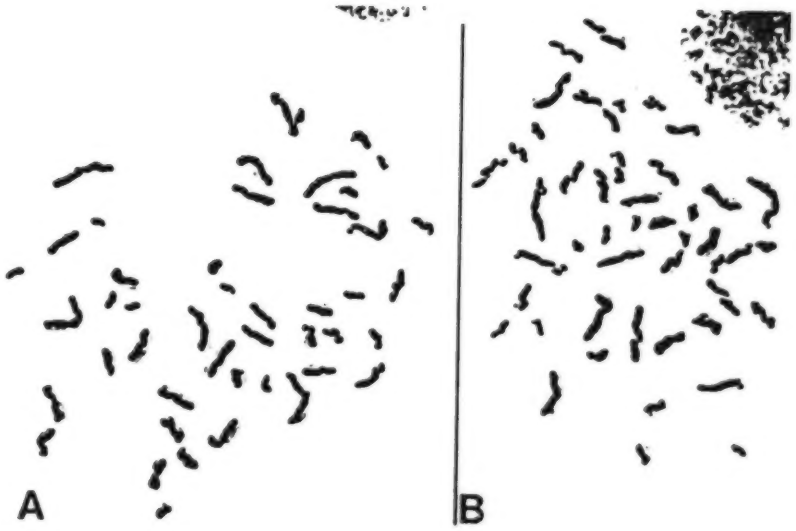

Figure 2. Human lymphocyte chromosomes substituted with BrdU and showing differential Giemsa staining. Cell A is a control example showing eight SCEs (evident as reciprocal discontinuities in staining). Cell B, which shows a moderate elevation in the incidence of SCE, is from a culture exposed to 0.1 µg/ml of vinyl carbamate. Approximately 40 SCEs are evident.

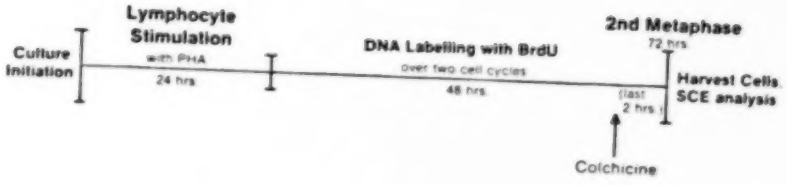

Figure 3. Basic steps involved in culturing human lymphocytes for SCE analysis.